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MUTAGEN AND ONCOGEN STUDY ON N-PHENYL-ALPHA-NAPHTHYLAMINE FINAL REPORT

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20795

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AEROSPACE MEDICAL RESEARCH LABORATORY
AEROSPACE MEDICAL DIVISION
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WRIGHT-PATTERSON AIR FORCE BASE, OHIO 45433

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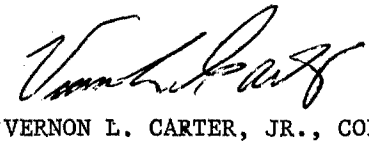

VERNON L. CARTER, JR., COLONEL, USAF, VC
Deputy Director
Toxic Hazards Division
Aerospace Medical Research Laboratory

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A three tier test was organized into a matrix of assays employing microbial cells, mammalian cells in culture, and in vivo experiments in mice. N-phenyl-alpha-naphthylamine did not exhibit mutagenic activity in any of the assays. A weak positive response for unscheduled DNA synthesis (UDS) in WI-38 cells was noted at a single dose level of the compound. This effect was reproduced in a repeat test of UDS. Because all other assays were negative and a good dose-related response for UDS in WI-38 cells was not obtained, the UDS data were viewed as a suggestion of activity that will require further

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PREFACE

This research was initiated by the Toxicology Branch, Toxic Hazards Division, Aerospace Medical Research Laboratory. Experiments were performed under Contract F33615-76-C-0515 by Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland 20795.

The experiments were conducted by David Brusick, Ph.D., and Dale W. Matheson, Ph.D., of Litton Bionetics, Inc., Kensington, Maryland 20795. Kenneth C. Back, Ph.D., was contract monitor for the Aerospace Medical Research Laboratory.

1. INTRODUCTION

The detection and subsequent confirmation of mutagenic substances capable of producing germ cell mutations requires a multifaceted testing program. The components of such a program should be able to detect both point mutations and chromosomal aberrations since these two classes of genetic alterations represent the types of transmissible mutations that are of concern to man. The tests included in a mutagenicity evaluation program for chemicals should not only be sensitive and reproducible, but also relevant to normal exposure and pharmacological conditions encountered in the environment. These latter two conditions are often difficult to achieve since good human model systems are lacking. It may be argued that if a single toxicologic end point, e.g., mutation, can be demonstrated in several different test species, then application of the response to a wide range of species, including man, can be made. Therefore, a mutagenicity evaluation program should contain a series of assays covering many phylogenetic levels.

We feel that the program conducted in this study offered as many of the essential test criteria as possible for an accurate evaluation of N-phenyl-alpha-naphthylamine for genetic activity. Selected tests from Tiers I, II, and III were organized into a matrix of assays employing microbial cells, mammalian cells in culture, and in vivo experiments in rats and mice.

Tests utilizing these organisms measured point mutations (forward and reverse), chromosomal aberrations, and mitotic recombinational events induced by acute and subchronic exposure to the test substance.

Figure 1 illustrates the composition of the test program prepared for the genetic evaluation of the test substance. A brief summary of each of the assays is listed as follows:

A. In Vitro Microbial Assays

In these assays, the test substance was evaluated for mutagenic and recombinogenic activity in strains of Salmonella and Saccharomyces, respectively. Metabolic activation of the compound was obtained by combining hepatic microsomes with the test system. Nonactivation and activation semiquantitative plate tests were conducted.

B. In Vitro Mutation Assay in Mammalian Cells

In this assay, the mutations were measured in cultured mouse cells (L5178Y). Both direct and in vitro activation assays were performed. The specific event detected by these cells

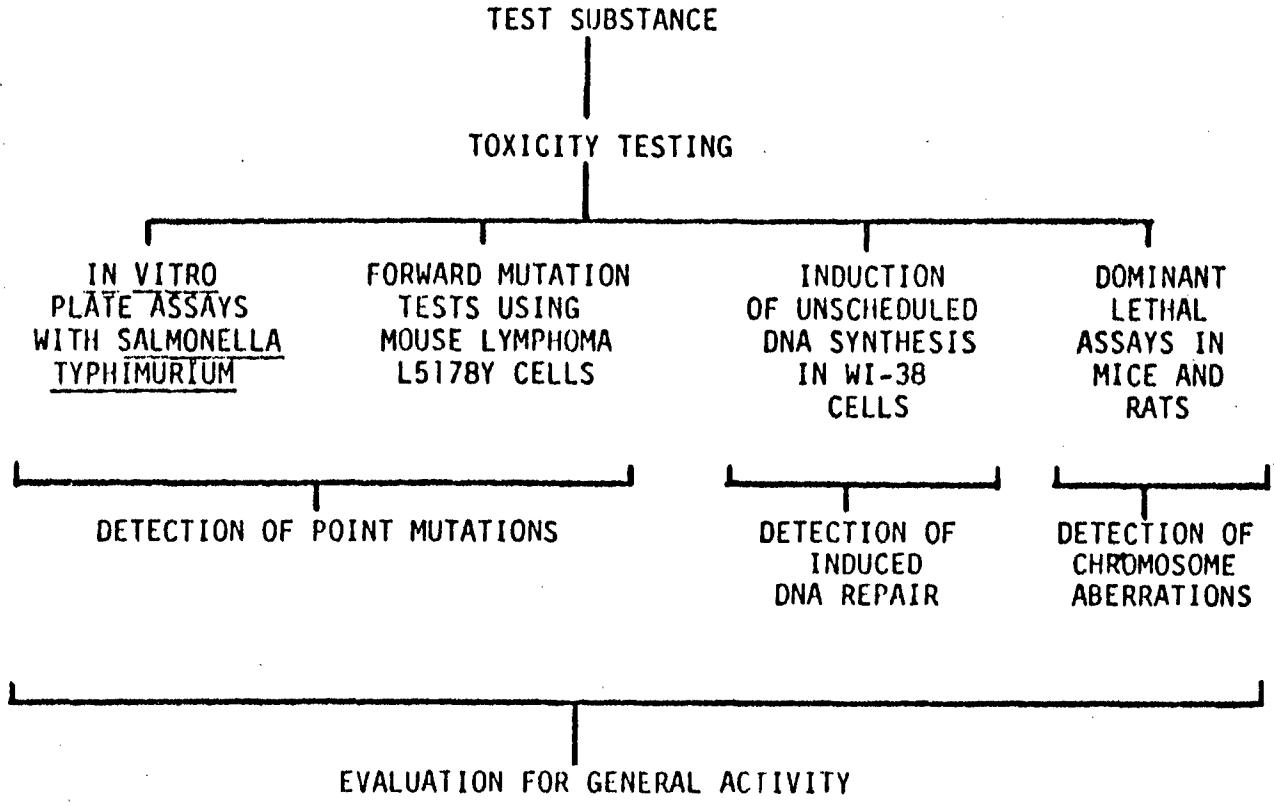

FIGURE 1

COMPOSITION OF THE GENETIC EVALUATION PROGRAM

was forward mutation at the thymidine kinase ($TK^{+/-} \rightarrow TK^{-/-}$) locus, which is an autosomal recessive trait. The combined *in vitro* tests from A and B gave a very sensitive measurement of the test substance's ability to induce point mutations and mitotic recombination.

C. Unscheduled DNA Synthesis

A second component of the *in vitro* mammalian cell assay system utilized the human diploid WI-38 strain of cells. This cell strain obtained from human embryonic lung was used to measure test chemical-induced DNA repair in cells not undergoing scheduled (S phase) DNA synthesis.

Normal DNA synthesis occurs in the S phase of the cell cycle with little or no synthesis occurring in any of the other phases (G_0, G_1, G_2, or M). The detection of significant DNA synthesis during these stages (UDS) is indicative of the stimulation of repair enzyme systems. Exposure of WI-38 cells to various forms of radiation or to chemicals known to be mutagenic or carcinogenic has resulted in the stimulation of UDS (1).

The detection of UDS in WI-38 cells involved exposure of the cells to the test chemical followed by the addition of tritiated thymidine (^{3}H-TdR) to the culture. If DNA damage has been induced, the ^{3}H-TdR will be incorporated during the repair of the DNA. This incorporation can be detected by scintillation counting.

D. Dominant Lethal Assay

This assay was designed to determine the ability of a compound to induce genetic damage to the germ cells of treated male mice and rats leading to fetal wastage. Chromosome aberrations, including breaks, rearrangements, and deletions, are believed to produce the dominant lethality. Male mice were exposed to several dose levels of the test compound for five days and then sequentially mated to two virgin untreated females each week over the period of spermatogenesis. At mid-pregnancy the females were killed and scored with respect to the number of living and dead implants as well as to the level of fertility. These results were then compared to data from control animals. This assay was designed to detect compounds capable of inducing chromosome alterations.

E. Background

Naphthylamine compounds are found among aromatic amines that exhibit mutagenic and carcinogenic activity. β-naphthlyamine (2NA) has been identified as both a carcinogen (9) and a mutagen (10).

Because the test agent is structurally related to chemicals that are established mutagens and carcinogens, and because of the excellent correlation between mutagenicity and carcinogenicity, mutagenesis studies might provide insight into potential toxicologic problems associated with the test agent.

2. MATERIALS

A. In Vitro Microbial Assays

The test chemical was examined in a series of microbial assays employing histidine-requiring mutants of Salmonella typhimurium. The assays were conducted so that the compounds was tested directly and in the presence of a mouse liver microsome activation system.

The compound was evaluated at a minimum of four dose levels under both test conditions with the highest dose level showing some evidence of toxicity. In addition to these tests, spot tests (5) were conducted with the Salmonella mutants plus additional strains of bacteria; S. typhimurium strain G-46 and E. coli strain WP_2uvrA^- (11).

1. Preparation of Tissue Homogenates and 9,000 x g Cell Fractions

Male mice (sufficient to provide the necessary quantities of tissues) were killed by cranial blow, decapitated, and bled. Organs were immediately dissected from the animal using aseptic techniques and placed in ice-cold 0.25 M sucrose buffered with Tris buffer at a pH of 7.4. Upon collection of the desired quantity of organs, they were washed twice with fresh buffered sucrose and completely homogenized with a motor-drive homogenizing unit at 4C. The whole organ homogenate obtained from this step was centrifuged for 20 minutes at 9,000 x g in a refrigerated centrifuge. The supernatant from the centrifuged sample was retained and frozen at -80C. Samples from these preparations were used for the activation studies.

2. Reaction Mixture

The following reaction mixture was employed in the activation tests:

Component	Final Concentration/ml
TPN (sodium salt)	6 µM
Isocitric acid	35 µM
Tris buffer, pH 7.4	28 µM
$MgCl_2$	2 µM
Homogenate fraction equivalent to 25 mg of wet tissue	

3. Solvent and Control Compounds

Preparation and dilution of test compounds were done in dimethylsulfoxide (DMSO). Positive control compounds were included as reference points and to ensure that the assay was functioning with known mutagens. Direct acting mutagens were employed in nonactivation assays and mutagens requiring microsomal activation were used in activation assays. The compounds and the concentrations employed are provided in the data tables.

4. Bacteria Cultures

Overnight cultures of Salmonella typhimurium G-46, TA-1535, TA-1537, TA-1538, TA-98, and TA-100 were employed along with E. coli strain WP_2uvrA^- and Saccharomyces cerevisiae strain D4. All cultures were monitored regularly for stability of markers and contamination.

B. In Vitro Mutation Assay in Mammalian Cells

The test chemical was tested for mutagenic activity in a forward mutation assay employing cultured mouse cells (L5178Y). The cell line is heterozygous for the Thymidine Kinase ($TK^{+/-}$) gene and the assay detects homozygous $TK^{-/-}$ mutant clones. The compounds were tested directly and in the presence of a mouse liver microsome activation system.

1. Preparation of Tissue Homogenates and 9,000 x g Cell Fractions: The activation system employed in this assay was the same as described for the Microbial Assays.

2. Reaction Mixture: The same reaction mixture as described for the Microbial assays was used for these studies.

3. Solvent and Control Compounds: Preparation of stock chemicals was done in DMSO. All dilutions of test chemicals were made in F_{10p} culture medium. Positive control mutagens active directly and requiring microsome activation were employed with all tests.

4. Cells and Media: $TK^{+/-}$ BUdR-sensitive L5178Y mouse lymphoma cells were used in this assay. Growth medium for this line consists of Fischer's mouse leukemia medium supplemented with 10% horse serum and sodium pyruvate (F_{10p}). Cloning medium consists of Fischer's medium plus 20% horse serum and agar (0.37%). Selective medium for $TK^{-/-}$ cells was prepared by adding BUdR to the cloning medium.

C. Unscheduled DNA Synthesis (UDS) Assay

Nondividing WI-38 cells were exposed to three concentrations of the test compound and ^{3}H-thymidine. Treatment was direct and under conditions of microsome activation. The amount of ^{3}H-thymidine incorporated into the DNA was measured by scintillation counting.

1. Preparation of Mouse Liver Microsomes: A 9,000 x g supernatant of mouse liver was prepared as described in the Microbial Assays. This supernatant was then centrifuged at 105,000 x g for 60 minutes and the pelleted microsomes resuspended in 0.25 M sucrose. This microsome preparation was added to the reaction mixture in place of the 9,000 x g cell fraction.

2. Reaction Mixture: The reaction mixture was the same as used in the Microbial Assays except purified microsomes replaced the 9,000 x g supernatant.

3. Solvent and Control Compounds: Any stock solutions of chemicals were prepared and diluted in DMSO. Positive control chemicals that act directly and require microsome activation were employed.

4. Cells and Media: Human diploid embryonic lung cells (WI-38) were obtained from Flow Laboratories and used in these assays. The growth medium (GM) employed was Eagle's minimal essential medium (EMEM) supplemented with 10% fetal calf serum. Step-down medium (SM) was amino acid depleted to reduce cell division, and hydroxyurea medium (HUM) was the medium used to inhibit S phase growth. All media were based on EMEM.

D. Dominant Lethal Assay (DLA)

The test chemical was tested in a standard mouse DLA. All animals were dosed by intraperitoneal injections over five consecutive days, rested for two days, and mated.

1. Animals: Seven- to eight-week-old male random bred mice (ICR, Flow) were used for treatment. Female mice of the same strain, age, and weight were used for the matings.

2. Animal Husbandry: Male mice were housed five to a cage while being dosed with the compound, and then housed separately with two females for mating. All animals were offered a 4% fat diet and water ad libitum. Water was acidified according to approved laboratory animal health standards.

Animals were identified by ear punch. Sanitary cages and bedding were used and changed two times per week at which times water containers were cleaned, sanitized and filled. Cages were repositioned on racks once a week, and the racks repositioned within rooms monthly. Personnel handling animals or working with animal facilities wear head and face masks as well as suitable garments. Individuals with respiratory or other overt infections are excluded from the animal facility.

3. Positive and Negative Control Chemicals: Triethylenemelamine (TEM) was administered intraperitoneally (IP) at a level of 0.3 mg/kg in 0.85% saline as a positive control. Negative control animals received an IP injection of the corn oil or water solvents.

E. Test Chemicals

The test sample was obtained from the United States Air Force. N-phenyl-alpha-naphthylamine (PANA) was a red-brown solid in pellet form. This compound was ground into fine crystals and dissolved in DMSO or corn oil.

3. METHODS

A. In Vitro Microbial Assays

Overnight cultures of S. typhimurium TA-1535, TA-1537, TA-1538, TA-98, TA-100, E. coli WP_2uvrA^-, and S. cerevisiae D4 were grown in complete broth. Approximately 10^8 cells from a culture were added to test tubes containing 2.0 ml of molten agar supplemented with biotin and a trace amount of histidine. Four dose levels of the test chemical were added to the appropriate tubes and the contents poured over selective medium. In activation tests 0.5 ml aliquots of the reaction mixture containing the microsomes were added to the tubes containing cells and chemical just prior to pouring onto the selective medium. After the overlays solidified, the plates were placed in a 37C incubator for 48 to 72 hours. The plates were then scored for the number of colonies growing in the agar overlay. Positive and solvent controls using both direct-acting mutagens and promutagens that required metabolic activation were run with each assay. Supplementary spot tests were also conducted according to the methods described by Ames et al. (12).

The data are presented in Table 1. Concentrations of the test and positive control chemicals are given in the data tables.

B. In Vitro Mutation Assay in Mammalian Cells

1. Toxicity

The solubility, toxicity, and doses for the test chemical were determined prior to screening. The effect of the chemical on the survival of the indicator cells was determined by exposing the cells to a wide range of chemical concentrations in complete growth medium. Toxicity was measured as loss in growth potential of the cells induced by a five-hour exposure to the chemical. Four doses were selected from the range of concentration by using the highest dose that showed no loss in growth potential as the penultimate dose and by bracketing this with one higher dose and two lower doses. Toxicity produced by chemical treatment was monitored during the experiment.

2. Test

a. Nonactivation assay

The procedure used is a modification of that reported by Clive and Spector (13). Prior to each treatment, cells were cleansed of spontaneous $TK^{-/-}$ by growing them in a medium containing thymidine, hypoxanthine, methotrexate, and glycine (THMG). This medium permits the survival of only those cells that produce the enzyme thymidine kinase, and can therefore utilize the exogenous thymidine from the medium. The test compound was added to the cleansed cells in growth medium at the predetermined doses for five hours. The mutagenized cells were washed, fed, and allowed to express in growth medium for three days. At the end of this expression period, $TK^{-/-}$ mutants were detected by cloning the cells in the selection medium for ten days. Surviving cell population were determined by plating diluted aliquots in nonselective growth medium.

b. Activation Assay

The activation assay differs from the nonactivation assay in the following manner only. Two and five tenths ml of the reaction mixture was added to 10 ml of growth medium. The desired number of cleansed cells was added to this mixture, and the flask was incubated on a rotary shaker for five hours. The incubation period was terminated by washing the cells twice with growth medium. The washed mutagenized cells were then allowed to express for three days and were cloned as indicated for the nonactivated cells.

c. Data Analysis

A mutation frequency for each test dose was determined by dividing the number of mutants/ml by the number of surviving cells/ml (adjusted to 10^{-4}) as indicated by plating efficiency. These data are presented in Table 2. Concentrations of the test and positive control chemicals are given in the data tables.

C. Unscheduled DNA Synthesis

1. Cell Preparation

Normal human diploid WI-38 cells were seeded at 5×10^5 cells in a 100 mm tissue culture dish and grown to confluency in GM. Once reaching confluency, the cells were switched to SM for 5 days. The contact inhibition imposed by confluency and the use of SM held the cells in a nonproliferating state.

2. Treatment

On the day of treatment, cells held in G_1 phase were placed in HUM. After 30 minutes this medium was replaced by 5 ml of HUM containing the control or test chemical and 1.0 µCi of ^{3}HTdR. Each treatment was at three concentrations. Exposure was terminated by washing the cells twice in cold BSS containing an excess of cold thymidine.

3. DNA Extraction and Measurement of ^{3}HTdR Incorporation

Treated plates were frozen at -20C until processed. After thawing, the cells on the 100 mm plate were covered with 2.5% SDS in 1 x SSC and scraped from the dish with a rubber policeman. The cells were washed and precipitated from the SDS by three changes of 95% ethanol and centrifuged at 10,000 x g. Additional lipid components were removed by extraction in ethanol ether at 70C. This pellet was washed in 70% ethanol, further incubated at 70C in 0.3N NaOH, and the DNA extracted in 50 µl 1N PCA at 70C. The DNA was separated into two 25 µl aliquots. One of these was dissolved in 10 ml of hydromix scintillation cocktail (Yorktown Co.) and counted in a Beckman Liquid Scintillation spectrometer. The second aliquot was added to 275 µl of 1N PCA and read at 260 nm in a Gilford spectrophotometer. The values were corrected for light scatter and converted to µg of DNA. Following liquid scintillation counting, the data were combined with the DNA extraction values and expressed as Disintegration Per Minute Per µg DNA (DPM/µg DNA).

4. Activation Assays

The activation tests were conducted according to the methods described above except that 0.62 ml of a purified microsome preparation (100,000 x g pellet) was added to the test mixture.

5. Dosage Determinations

Doses were determined from preliminary toxicity tests in which cells were seeded in 16 mm wells (Linbro plate). A wide range of concentrations were tested in the wells, and toxicity was monitored visually by altered cell morphology and adhesion. The three doses used in the experiments were selected.

The results of these tests are given in Table 3. The concentrations of test and control compounds are given in the data tables.

D. Dominant Lethal Assay

The dominant lethal assay is designed to assess the ability of the test compound or its metabolic products to reach the testes of treated male animals and induce genetic activity in the developing gametes during spermatogenesis.

N-phenyl-alpha naphthylamine was administered to male mice weighing 30 ± 2.5 gms.

1. Stock Solutions

The compound was prepared daily from stock solutions. N-phenyl-alpha-naphthylamine pellets were ground with a mortor and pestle, weighed, and dissolved in corn oil at room temperature.

2. Compound Administration

Dosages were determined from LD50 data supplied by the contract monitor with a high dose of 1/10 the LD50, an intermediate dose at 1/3 the high level, and a low dose of 1/10 the high level. Compound was injected intraperitoneally into each animal daily for five days. All dosages and routes of administration were determined in consultation with Dr. Kenneth Back of the United States Air Force.

Calculated dosages are as follows:

N-phenyl-alpha-naphthylamine

Mice

LD50	1231 mg/kg
High 1/10 LD50	500 mg/kg*
Int. 1/30 LD50	166 mg/kg
Low 1/100 LD50	50 mg/kg

*This dose was selected as the high dose by the contract monitor.

3. Animal Husbandry

Ten male mice were housed five animals to a cage during the five days of dosing. After two days of rest, each male was caged with two virgin females from Monday through Friday. This sequence was repeated weekly with two new females each week for eight weeks. Fourteen days from the midweek in which they were caged with the males, females were sacrificed, dissected, and the number of dead, living, and total embryos in the uterus recorded on the standard forms. These data were statistically analyzed for indications of dominant lethality, and compared with control data for significance.

4. Data

The results of the Dominant Lethal Assay are given in Tables 4-9.

4. RESULTS

The results of the genetic studies are presented in the following series of tables:

TABLE 1

RESULTS FROM MICROBIAL ASSAYS EVALUATING THE GENETIC ACTIVITY OF PANA

Concentration (µl/plate)	Revertants Per Plate with Indicator Strain						
	TA-1535	TA-1537	TA-1538	TA-98	TA-100	D4	WP_2 *uvrA*-
Nonactivation							
Solvent Control	23(-)	18(-)	21(-)	17(-)	117(-)	30(-)	(-)
Positive Control	>10^3(+)	>10^3(+)	325(+)	440(+)	>10^3(+)	172(+)	(+)
PANA							
0.5	25	18	20	12	134	28	-
5.0	20	13	15	13	113	39	-
50.0	12	11	18	12	126	41	-
250.0	22	30	21	10	92	27	-
500.0	-(-)	11(-)	-(-)	-(-)	-(-)	-(-)	(-)
Activation							
Solvent Control	32(-)	21(-)	30(-)	72(-)	166(-)	33(-)	(-)
Positive Control	210(+)	240(+)	>10^3(+)	>10^3(+)	>10^3(+)	-	(+)
PANA							
0.5	33	28	27	44	163	35	-
5.0	35	30	30	41	191	34	-
50.0	23	35	12	38	181	37	-
250.0	37	37	30	44	158	33	-
500.0	-(-)	27(-)	-(-)	-(-)	-(-)	-(-)	(-)

( ) = Results of qualitative spot test (+) = positive response
(-) = negative response

TABLE 1A

A COMPARISON OF THE MUTAGENIC ACTIVITY 2-NAPHTHYLAMINE AND P-ALPHA-NAPHTHYLAMINE IN PLATE ASSAYS WITH TA-1535 AND TA-100

Test Compound	Concentration Per Plate	Revertants Per Plate[a]	
		TA-1535	TA-100
Solvent Control	-	18	166
2-Naphthylamine	10 µg	126	154
	20 µg	189	269
	50 µg	172	268
	100 µg	174	224
Solvent Control	-	22	113
PANA	10 µg	14	146
	20 µg	13	124
	50 µg	11	108
	100 µg	10	161

[a]Test conditions included rat liver microsome activation.

KEY

MOUSE LYMPHOMA ASSAY TABLE

COLUMN		
A, B, C, D	Day	= Expression day cell counts ($\times 10^6$)
E	ΔGS	= Represents cell population growth during expression. The value is obtained by subtracting the Day 1 counts from the terminal day counts.
F	%GS	= Percent suspension growth is obtained by expressing the ΔGS values for treated cells as a percent of the ΔGS for the negative controls $\frac{\text{E treated}}{\text{E control}} \times 100$
G	MC	= Mutant counts. The total number of colonies counted in the BUdR plates.
H	VC	= Viable counts. The total number of colonies counted in the VC plates.
I	%CE	= Cloning efficiency $\frac{\text{VC counts in treated cultures}}{\text{VC counts in control cultures}} \times 100$
J	GF	= Growth factor $\frac{\text{Percent suspension growth (column F)} \times \text{Percent clonal growth (column I)}}{100}$
K	MF($\times 10^{-4}$)	= Mutation frequency $\frac{\text{MC counts (column G)}}{\text{VC counts (column H)}} \times 10^{-4}$

TABLE 2

RESULTS OF THE MOUSE LYMPHOMA MUTAGENICITY ASSAY FOR PANA

Test	A Day 1	B 2	C 3	D 4	E ΔGS	F % GS	G MC	H VC	I % CE	J GF	K MF(10^{-4})
Nonactivation											
Solvent Control	1.5	-	11.1	-	9.6	100	89	191	100	100	0.5
Positive Control	1.6	-	2.3	-	0.7	7	288	7	4	0.3	41.1
PANA											
0.5 µg/ml	3.1	-	11.2	-	8.1	84	68	214	112	94	0.3
5.0 µg/ml	2.7	-	8.6	-	5.9	61	5	300	157	95	0.02
10.0 µg/ml	2.6	-	10.9	-	8.3	86	50	202	105	122	0.3
25.0 µg/ml	3.4	-	12.4	-	9.0	93	57	215	112	104	0.3
Activation											
Solvent Control	1.1	-	7.2	-	6.1	100	58	229	100	100	0.3
Positive Control	1.5	-	2.7	-	0.2	3	184	8	3	0.9	2.3
PANA											
0.005 µg/ml	3.3	-	17.8	-	14.5	246	8	56	69	170	0.1
0.01 µg/ml	3.1	-	15.6	-	12.5	212	3	58	72	152	0.05
0.05 µg/ml	2.8	-	6.5	-	3.7	62	32	70	86	54	0.5
0.1 µg/ml	0.9	-	1.7	-	0.8	14	6	36	44	6	0.2

TABLE 3

MEASUREMENT OF UDS IN WI-38 CELLS TREATED WITH PANA

Test	Concentration (μg/ml)	DNA(μg)	DPM	Activity Index[a]	Percent of Control[b]
Nonactivation					
Solvent Control	-	9.02	76	8.4	-
Positive Control	MNNG (10μg/ml)	1.76	86	48.9	582
PANA	10	6.00	79	13.2	157
	50	3.51	67	19.1	227
	100	Toxic			
Activation					
Solvent Control	-	13.64	78	5.7	-
Positive Control	2AAF (30μg/ml)	2.45	60	24.5	430
PANA	5	19.48	80	4.1	72
	10	9.34	55	5.9	104
	50	18.24	104	5.7	100

[a]Activity Index = DPM/μg DNA (DPM = Disintegrations/minute)

[b]Percent of Control = $\frac{\text{Activity Index Treated}}{\text{Activity Index Control}} \times 100$

TABLE 3A

THE ACTIVITY OF PANA IN TESTS DETECTING THE STIMULATION OF UDS IN WI-38 CELLS

Test	Treatment Concentration	DPM	µg DNA	Activity Index (DPM/µg DNA)	Percent of Control[b]
Nonactivation					
Solvent Control	-	162	42	3.83	-
Positive Control	(MNNG) 10 µg/ml	460	34	13.70	358
	PANA 5 µg/ml	439	32	13.90	363
	PANA 10 µg/ml	297	43	6.90	180
	PANA 50 µg/ml	514	45	11.40	296
Activation[a]					
Solvent Control	-	153	44	3.51	-
Positive Control	(AAF) 20 µg/ml	518	38	13.74	391
	PANA 5 µg/ml	219	42	5.24	149
	PANA 10 µg/ml	459	34	13.50	385
	PANA 50 µg/ml	184	45	4.06	116

[a]The activation system consisted of purified microsomes from mouse liver plus associated cofactors.

[b]Percent of Control = $\frac{\text{Activity Index Treated}}{\text{Activity Index Control}} \times 100$

TABLE 4
COMPOUND PANA STUDY SUBACUTE/MICE

FERTILITY INDEX

LOG DOSE	ARITH DOSE	WEEK	NEGATIVE CONTROL	DOSE LEVEL 50.0 MG/KG	DOSE LEVEL 166.0 MG/KG	DOSE LEVEL 500.0 MG/KG	POSITIVE CONTROL
		1	7/ 20=0.35	4/ 19=0.21	6/ 20=0.30	3/ 20=0.15	2/ 20=0.10
		2	11/ 20=0.55	11/ 20=0.55	7/ 20=0.35	11/ 20=0.55	5/ 20=0.25
		3	10/ 20=0.50	6/ 19=0.32	6/ 20=0.30	8/ 20=0.40	5/ 20=0.25
		4	12/ 20=0.60	8/ 20=0.40	6/ 20=0.30	9/ 20=0.45	14/ 20=0.70
		5	5/ 17=0.29	10/ 20=0.50	4/ 20=0.20	7/ 19=0.37	1/ 20=0.05*
		6	16/ 20=0.80	13/ 20=0.65	12/ 20=0.60	11/ 20=0.55	13/ 16=0.81
		7	13/ 20=0.65	3/ 20=0.15**	10/ 20=0.50	10/ 20=0.50	11/ 20=0.55
$	$	8	11/ 20=0.55	10/ 20=0.50	6/ 20=0.30	5/ 20=0.25	10/ 20=0.50

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

ONE $,* = SIGNIFICANT AT P LESS THAN 0.05
TWO $,* = SIGNIFICANT AT P LESS THAN 0.01

* SIGNIFICANTLY DIFFERENT FROM CONTROL
$ SIGNIFICANT LINEAR RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE 5

COMPOUND PANA STUDY SUBACUTE/MICE

AVERAGE NUMBER OF IMPLANTATIONS PER PREGNANT FEMALE

LOG DOSE	ARITH DOSE	WEEK	NEGATIVE CONTROL	DOSE LEVEL 50.0 MG/KG	DOSE LEVEL 166.0 MG/KG	DOSE LEVEL 500.0 MG/KG	POSITIVE CONTROL
		1	72/ 7=10.3	45/ 4=11.3	64/ 6=10.7	34/ 3=11.3	16/ 2= 8.0**@@D
		2	118/ 11=10.7	126/ 11=11.5	77/ 7=11.0	135/ 11=12.3	50/ 5=10.0
		3	117/ 10=11.7	75/ 6=12.5	84/ 6=14.0@I	97/ 8=12.1	46/ 5= 9.2
		4	138/ 12=11.5	91/ 8=11.4	61/ 6=10.2	106/ 9=11.8	160/ 14=11.4
	$	5	59/ 5=11.8	106/ 10=10.6	44/ 4=11.0	60/ 7= 8.6@D	12/ 1=12.0
		6	204/ 16=12.8	143/ 13=11.0@D	148/ 12=12.3	142/ 11=12.9	183/ 13=14.1
		7	139/ 13=10.7	28/ 3= 9.3	112/ 10=11.2	116/ 10=11.6	117/ 11=10.6
		8	135/ 11=12.3	125/ 10=12.5	74/ 6=12.3	68/ 5=13.6	132/ 10=13.2

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

& AND * = TWO-TAILED TEST
$ AND @ = ONE-TAILED TEST

ONE $,&,@,* = SIGNIFICANT AT P LESS THAN 0.05
TWO $,&,@,* = SIGNIFICANT AT P LESS THAN 0.01

*,@ SIGNIFICANTLY DIFFERENT FROM CONTROL
&,$ SIGNIFICANT RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE 6

COMPOUND PANA STUDY SUBACUTE/MICE

AVERAGE RESORPTIONS (DEAD IMPLANTS) PER PREGNANT FEMALE

LOG DOSE	ARITH DOSE	WEEK	NEGATIVE CONTROL	DOSE LEVEL 50.0 MG/KG	DOSE LEVEL 166.0 MG/KG	DOSE LEVEL 500.0 MG/KG	POSITIVE CONTROL
		1	0/ 7=0.0	1/ 4=0.25	1/ 6=0.17	0/ 3=0.0	7/ 2=3.50**@@I
		2	3/ 11=0.27	3/ 11=0.27	5/ 7=0.71	5/ 11=0.45	39/ 5=7.80**@@I
		3	7/ 10=0.70	1/ 6=0.17	0/ 6=0.0 *@@D	5/ 8=0.63	20/ 5=4.00**@@I
		4	4/ 12=0.33	5/ 8=0.63	5/ 6=0.83	1/ 9=0.11	8/ 14=0.57
		5	4/ 5=0.80	5/ 10=0.50	1/ 4=0.25	3/ 7=0.43	0/ 1=0.0
		6	4/ 16=0.25	3/ 13=0.23	9/ 12=0.75*@I	4/ 11=0.36	7/ 13=0.54
		7	5/ 13=0.38	1/ 3=0.33	6/ 10=0.60	7/ 10=0.70	4/ 11=0.36
		8	7/ 11=0.64	8/ 10=0.80	4/ 6=0.67	2/ 5=0.40	1/ 10=0.10*@D

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

& AND * = TWO-TAILED TEST
$ AND @ = ONE-TAILED TEST

ONE $,&,@,* = SIGNIFICANT AT P LESS THAN 0.05
TWO $,&,@,* = SIGNIFICANT AT P LESS THAN 0.01

*,@ SIGNIFICANTLY DIFFERENT FROM CONTROL
&,$ SIGNIFICANT RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE 7
COMPOUND PANA STUDY SUBACUTE/MICE

PROPORTION OF FEMALES WITH ONE OR MORE DEAD IMPLANTATIONS

LOG DOSE	ARITH DOSE	WEEK	NEGATIVE CONTROL	DOSE LEVEL 50.0 MG/KG	DOSE LEVEL 166.0 MG/KG	DOSE LEVEL 500.0 MG/KG	POSITIVE CONTROL
		1	0/ 7=0.0	1/ 4=0.25	1/ 6=0.17	0/ 3=0.0	2/ 2=1.00**
		2	2/ 11=0.18	2/ 11=0.18	3/ 7=0.43	4/ 11=0.36	5/ 5=1.00**
		3	5/ 10=0.50	1/ 6=0.17	0/ 6=0.0 *	5/ 8=0.63	5/ 5=1.00
		4	4/ 12=0.33	5/ 8=0.63	2/ 6=0.33	1/ 9=0.11	6/ 14=0.43
		5	2/ 5=0.40	4/ 10=0.40	1/ 4=0.25	3/ 7=0.43	0/ 1=0.0
		6	2/ 16=0.13	2/ 13=0.15	7/ 12=0.58*	3/ 11=0.27	5/ 13=0.38
		7	3/ 13=0.23	1/ 3=0.33	5/ 10=0.50	5/ 10=0.50	3/ 11=0.27
		8	6/ 11=0.55	7/ 10=0.70	3/ 6=0.50	2/ 5=0.40	1/ 10=0.10*

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

ONE $,* = SIGNIFICANT AT P LESS THAN 0.05
TWO $,* = SIGNIFICANT AT P LESS THAN 0.01

* SIGNIFICANTLY DIFFERENT FROM CONTROL
$ SIGNIFICANT LINEAR RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE 8

COMPOUND PANA STUDY SUBACUTE/MICE

PORPORTION OF FEMALES WITH TWO OR MORE DEAD IMPLANTATIONS

LOG DOSE	ARITH DOSE	WEEK	NEGATIVE CONTROL	DOSE LEVEL 50.0 MG/KG	DOSE LEVEL 166.0 MG/KG	DOSE LEVEL 500.0 MG/KG	POSITIVE CONTROL
		1	0/ 7=0.0	0/ 4=0.0	0/ 6=0.0	0/ 3=0.0	2/ 2=1.00**
		2	1/ 11=0.09	1/ 11=0.09	2/ 7=0.29	1/ 11=0.09	5/ 5=1.00**
		3	1/ 10=0.10	0/ 6=0.0	0/ 6=0.0	0/ 8=0.0	4/ 5=0.80**
		4	0/ 12=0.0	0/ 8=0.0	2/ 6=0.33*	0/ 9=0.0	2/ 14=0.14
		5	1/ 5=0.20	1/ 10=0.10	0/ 4=0.0	0/ 7=0.0	0/ 1=0.0
		6	1/ 16=0.06	1/ 13=0.08	2/ 12=0.17	1/ 11=0.09	2/ 13=0.15
		7	2/ 13=0.15	0/ 3=0.0	1/ 10=0.10	2/ 10=0.20	1/ 11=0.09
		8	1/ 11=0.09	1/ 10=0.10	1/ 6=0.17	0/ 5=0.0	0/ 10=0.0

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

ONE $,* = SIGNIFICANT AT P LESS THAN 0.05
TWO $,* = SIGNIFICANT AT P LESS THAN 0.01

* SIGNIFICANTLY DIFFERENT FROM CONTROL
$ SIGNIFICANT LINEAR RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE 9

COMPOUND PANA STUDY SUBACUTE/MICE

DEAD INPLANTS / TOTAL IMPLANTS

WEEK	NEGATIVE CONTROL	DOSE LEVEL 50.0 MG/KG	DOSE LEVEL 166.0 MG/KG	DOSE LEVEL 500.0 MG/KG	POSITIVE CONTROL
1	0/ 72=0.0	1/ 45=0.02	1/ 64=0.02	0/ 34=0.0	7/ 16=0.44*@@I
2	3/118=0.03	3/126=0.02	5/ 77=0.06	5/135=0.04	39/ 50=0.78**@@I
3	7/117=0.06	1/ 75=0.01	0/ 84=0.0 **@@D	5/ 97=0.05	20/ 46=0.43**@@I
4	4/138=0.03	5/ 91=0.05	5/ 61=0.08	1/106=0.01	8/160=0.05
5	4/ 59=0.07	5/106=0.05	1/ 44=0.02	3/ 60=0.05	0/ 12=0.0
6	4/204=0.02	3/143=0.02	9/148=0.06*@I	4/142=0.03	7/183=0.04
7	5/139=0.04	1/ 28=0.04	6/112=0.05	7/116=0.06	4/117=0.03
8	7/135=0.05	8/125=0.06	4/ 74=0.05	2/ 68=0.03	1/132=0.01*@D

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT DIFFERENCES USING THE HISTORICAL CONTROL GROUP

* = TWO-TAILED TEST
@ = ONE-TAILED TEST

ONE *,@ = SIGNIFICANT AT P LESS THAN 0.05
TWO *,@ = SIGNIFICANT AT P LESS THAN 0.01

*,@ SIGNIFICANTLY DIFFERENT FROM CONTROL

E. Conclusions

Except for the nonactivation results with PANA that appeared to stimulate UDS in WI-38 cells, the data from the other test systems were all negative. Although the result would normally be considered aberrant, it cannot be completely dismissed without additional observations. The overall conclusions from the accumulated data were that there was no consistent pattern of responses indicative of genetic activity by PANA and that the compound did not exhibit biological activity consistent with the most known mutagens and carcinogens. However, the reproducibility of the UDS response indicates the need for further investigation.

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5. INTERPRETATIONS AND CONCLUSIONS

N-phenyl-alpha-naphthylamine (PANA) was evaluated for its genetic activity and ability to stimulate DNA repair using a battery of *in vitro* and *in vivo* assays.

A. Microbial Assays (Tables 1 and 1A)

The results of these tests were negative. The same procedures using β-naphthylamine as the test compound were positive for strain TA-1535. Thus PANA does not have β-naphthylamine-like mutagenic properties.

B. Mouse Lymphoma Assay (Table 2)

The results of these tests were negative.

C. UDS Assay in WI-38 Cells (Tables 3 and 3A)

Data from nonactivation tests show a weak positive response at 50 μg/ml. The next higher dose used, 100 μg/ml, was toxic for the cells and a measurement of UDS could not be made. The data from the activation tests were negative. The positive results might have been aberrant since they were not observed in the activation test; or, the activation system may have detoxified or quenched out the potential activity observed in nonactivation tests. A repeat test for UDS (Table 3A) also showed activity in nonactivation tests. One point in the activation tests also appeared positive. The lack of a clear dose-related response reduced confidence in the effect but indicates further testing is warranted.

D. Dominant Lethal Assay in Mice (Tables 4-9)

No significant trends indicating the induction of dominant lethality by PANA were observed in this study. Isolated cases of apparent dominant lethality, such as shown by the intermediate dose of week 6, were not part of a dose-related trend and had dead implant/total implant ratios that fell within the range of all negative control groups. TEM gave a strong positive response during weeks 1 through 3.

A decrease in reproductive performance was noted at weeks 1 and 5, but this was not compound related since it was also observed in the negative control animals. The reason for this reduced fertility at week 5 was not determined. Reduced fertility during the first week of mating is normal.

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